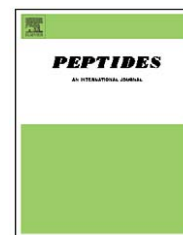


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Structure-activity relationships for in vitro diuretic activity of CAP2b in the housefly

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ABSTRACT

A series of truncated and Ala-replacement analogs of the peptide Manse-CAP2b (pELYAFPRV-NH₂) were assayed for diuretic activity on Malpighian tubules of the housefly *Musca domestica* (*M. domestica*). The C-terminal hexapeptide proved to be the active core, the minimum sequence required to retain significant diuretic activity. However, full activity required the C-terminal heptapeptide, which was equipotent with the most active of the native housefly CAP2b peptides. Replacement of Arg⁷ and Val⁸ with Ala led to inactivity and a large 70-fold drop in potency, respectively, indicating that these were critical residues. The Leu² was semicritical, where a six-fold loss in potency was observed. Conversely, the replacement of all other residues with Ala led to much smaller effects on potency and these positions were considered to be noncritical. This structure-activity relationship data can aid in the design of mimetic agonist/antagonist analogs of this diuretic peptide family with enhanced biostability and bioavailability, as tools for arthropod endocrinologists and as potential pest management agents capable of disrupting the water balance in pest flies.

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1. Introduction

It is well established that neuropeptides are key factors in controlling primary urine production in Malpighian tubules (CRF-like peptides, insect kinins, CAPA peptides) and fluid reabsorption from the hindgut (ion transport peptide (ITP)) [1,29]. CAP2b (pELYAFPRVa), a CAPA peptide (also included in the literature as a member of the periviscerokinins (PVK) family), was first identified as a cardioacceleratory factor in the moth *Manduca sexta* [11]. CAPA peptides, including CAP2b, demonstrate diuretic effects on the Malpighian tubules of a number of flies, including the fruit fly *Drosophila melanogaster*, the housefly *Musca domestica* (*M. domestica*), and the stable fly *Stomoxys calcitrans* [1,2–5,9,10,15,18,21,22,24–26,28]. By contrast,

bioassays on the bug *Rhodnius prolixus* with *Manduca* CAP2b [23,29] indicated that native CAPA peptides might reduce secretion by Malpighian tubules and thus show antidiuretic effects. These peptides are putative hormones typical of the neurosecretory system in the abdominal ventral nerve cord (VNC) of insects. The CAPA gene, which encodes for two or more CAP2b/PVKs and a single pyrokinin (PK), is known from a number of holometabolous insects, including *D. melanogaster* [12,13] and *M. sexta* [14]. Expressed in median neurosecretory neurons of abdominal ganglia, these putative peptide hormones may be released into the hemolymph via perisymphatic organs (PSOs) which are segmentally reiterated neurohemal organs of the abdominal ganglia [7,8]. Larval PSOs of cycloraphous Diptera, however, become incorporated into

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the dorsal ganglionic sheath [20,30,31] during metamorphosis. Direct analysis of abdominal dorsal sheath tissues via MALDI-TOF/TOF mass spectrometry led to the identification of CAP2b/PVK peptides native to the housefly *M. domestica* [18,26,32] and three other species of flies [18,19]. The housefly CAP2b/PVK sequences were recently determined to be AGGTSGLYAFPRVa (Musdo-CAP2b/PVK-1) and ASLFNAPRVa (Musdo-CAP2b/PVK-2) [18]. The octapeptide CAP2b shares a common C-terminal heptapeptide sequence (LYAFPRVa) with the most active housefly CAP2b sequence, Musdo-CAP2b-1, revealing the likely reason that the *Manduca* peptide demonstrates relatively potent diuretic activity in the heterologous housefly Malpighian tubule fluid secretion bioassay.

In this study, we use *Manduca* CAP2b as a model of Musdo-CAP2b-1 and evaluate a series of truncated and Ala-replacement CAP2b analogs in a housefly Malpighian tubule fluid secretion bioassay to identify the active core and those residues most critical to activity.

2. Materials and methods

2.1. Insects

Housefly (*M. domestica*) larvae were obtained from a commercial fish bait supplier. They were held in the laboratory until they pupated and were then moved to a constant temperature room (28 °C; 12 L:12 D light:dark cycle). Flies that emerged within 12 h of one another were kept as separate age cohorts and provided with water and sucrose ad libitum. All of the experiments described in this paper used 3–4 day-old adult female flies.

2.2. Peptide synthesis

The CAP2b analogs were synthesized via Fmoc methodology on Rink Amide resin (Novabiochem, San Diego, CA) using Fmoc protected amino acids (Advanced Chemtech, Louisville, KY) on an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA) under previously described conditions [17]. Crude products were purified on a Waters C₁₈ Sep Pak cartridge and a Delta Pak C₁₈ reverse-phase column (8 mm × 100 mm, 15 µm particle size, 100 Å pore size) on a Waters 510 HPLC controlled with a Millennium 2010 chromatography manager system (Waters, Milford, MA) with detection at 214 nm at ambient temperature. Solvent A = 0.1% aqueous trifluoroacetic acid (TFA); Solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Conditions: initial solvent consisting of 20% B was followed by the Waters linear program to 100% B over 40 min; flow rate, 2.0 ml/min. Delta-Pak C-18 retention times: Manse-CAP2b[2–8], 9.0 min; Manse-CAP2b[3–8], 7.5 min; Manse-CAP2b[4–8], 5.0 min; Manse-CAP2b[5–8], 4.5 min; Manse-CAP2b[Ala¹], 10.5 min; Manse-CAP2b[Ala²], 9.0 min; Manse-CAP2b[Ala³], 4.5 min; Manse-CAP2b[Ala⁵], 7.5 min; Manse-CAP2b[Ala⁶], 12.5 min; Manse-CAP2b[Ala⁷], 10.5 min; Manse-CAP2b[Ala⁸], 9.0 min. The peptides were further purified on a Waters Protein Pak I125 column (7.8 mm × 300 mm)(Milligen Corp., Milford, MA). Conditions: flow rate: 2.0 ml/min; isocratic with solvent consisting of 80% acetonitrile made to 0.01% TFA. WatPro retention times: Manse-CAP2b[2–8], 7.0 min; Manse-CAP2b[3–8], 8.0 min;

Manse-CAP2b[4–8], 11.5 min; Manse-CAP2b[5–8], 10.5 min; Manse-CAP2b[Ala¹], 15.5 min; Manse-CAP2b[Ala²], 15.0 min; Manse-CAP2b[Ala³], 13.5 min; Manse-CAP2b[Ala⁵], 16.5 min; Manse-CAP2b[Ala⁶], 11.0 min; Manse-CAP2b[Ala⁷], 5.5 min; Manse-CAP2b[Ala⁸], 15.0 min. Amino acid analysis was carried out under previously reported conditions [17] and used to quantify the peptide and to confirm identity, leading to the following analyses: Manse-CAP2b[2–8]: A[1.0], F[1.0], L[1.0], P[1.0], R[1.0], Y[1.0], V[1.0]; Manse-CAP2b[3–8]: A[1.0], F[1.0], P[1.1], R[1.0], Y[1.0], V[1.0]; Manse-CAP2b[4–8]: A[0.8], F[1.0], P[1.0], R[1.0], V[0.9]; Manse-CAP2b[5–8]: F[1.0], P[1.0], R[1.0], V[1.0]; Manse-CAP2b[Ala¹]: A[1.9], F[1.0], L[1.0], P[1.0], R[1.0], Y[1.1], V[0.9]; Manse-CAP2b[Ala²]: A[2.0], E[0.9], F[1.0], P[0.9], R[1.0], Y[1.1], V[1.0]; Manse-CAP2b[Ala³]: A[2.0], E[0.9], F[1.0], L[1.0], P[1.0], R[1.0], V[0.9]; Manse-CAP2b[Ala⁵]: A[2.0], E[0.8], L[1.0], P[0.9], R[1.0], Y[1.2], V[0.9]; Manse-CAP2b[Ala⁶]: A[2.0], E[1.0], F[1.0], L[0.9], R[1.0], Y[1.0], V[1.0]; Manse-CAP2b[Ala⁷]: A[2.1], E[0.7], F[1.0], L[1.1], P[1.1], Y[0.8], V[1.0]; Manse-CAP2b[Ala⁸]: A[2.0], E[1.0], F[1.0], L[1.0], P[0.9], R[1.0], Y[1.0]. The identities of the peptide analogs were confirmed via MALDI-TOF-MS on a Kratos Kompact Probe MALDI-TOF MS machine (Kratos Analytical, Ltd., Manchester, UK) with the presence of the following molecular ions ($M + H^+$): Manse-CAP2b[2–8], 864.9 [$M + H^+$]; Manse-CAP2b[3–8], 751.5 [$M + H^+$]; Manse-CAP2b[4–8], 588.6 [$M + H^+$]; Manse-CAP2b[5–8], 517.6 [$M + H^+$]; Manse-CAP2b[Ala¹], 936.8 [$M + H^+$]; Manse-CAP2b[Ala²], 933.8 [$M + H^+$]; Manse-CAP2b[Ala³], 883.9 [$M + H^+$]; Manse-CAP2b[Ala⁵], 899.5 [$M + H^+$]; Manse-CAP2b[Ala⁶], 949.3 [$M + H^+$]; Manse-CAP2b[Ala⁷], 890.6 [$M + H^+$]; Manse-CAP2b[Ala⁸], 947.7 [$M + H^+$].

2.3. Isolated housefly Malpighian tubule preparations

Fluid secretion from isolated housefly Malpighian tubules was measured using the “Ramsay assay” as previously described [10]. Tubules were removed from 3 to 4 day post-emergent adult female flies. Flies were dissected under *Musca* saline [10] and both anterior and posterior tubules were transferred to small (10 µl) drops of bathing fluid (a 1:1 mixture of saline and Schneider’s *Drosophila* medium) beneath water-saturated liquid paraffin in a Sylgard™ lined Petri dish. The tubules were allowed to equilibrate for 1 h before being challenged with test peptides. Urine escaped from the cut end of the tubule, which was pulled out into the liquid paraffin. Drops of urine were collected at 15 min intervals and their diameter (d) measured as they rested on the Sylgard base of the Petri dish using a Wild digital (MMS235) eyepiece micrometer. Urine volume was calculated as $\pi d^3/6$ and the rate of secretion obtained by dividing the secreted volume by the collection period. Data were normalized by expressing the increase in fluid secretion as a percentage of the response to 10 nM Musdo-K [16], which was added to all tubules at the end of each experiment. Dose-response curves were prepared using the computer program GraphPad Prism version 4.02 (GraphPad Software, San Diego, CA).

3. Results

A list of the fluid secretion activity of the two CAP2b/PVK peptides native to the housefly, Manse-CAP2b, Manse-CAP2b

Table 1 – Fluid secretion activity of CAP2b/PVK peptides and analogs on housefly (*M. domestica*) Malpighian tubules

Peptide	Sequence	Fluid secretion ^a	
		EC ₅₀ (nM) (95% CL)	Maximal response (%)
Musdo-CAP2b/PVK-1	AGGTSGLYAFPRVa	9 (7.2–11.1) [6]	100
Musdo-CAP2b/PVK-2	ASLFNAPRVa	102 (96–109) [6]	100
Manse-CAP2b	pELYAFPRVa	53 (39–72) [6]	
Manse-CAP2b[2–8]	LYAFPRVa	8 (3–22)	100
Manse-CAP2b[3–8]	YAFPRVa	428 (298–613)	100
Manse-CAP2b[4–8]	AFPRVa	Tr	
Manse-CAP2b[5–8]	FPRVa	Tr	
Manse-CAP2b[Ala ¹]	ALYAFPRVa	23 (11–46)	100
Manse-CAP2b[Ala ²]	pEAYAFPRVa	373 (255–544)	80
Manse-CAP2b[Ala ³]	pELAYAFPRVa	33 (10–105)	100
Manse-CAP2b[Ala ⁵]	pELYAAPRVa	115 (27–485)	70
Manse-CAP2b[Ala ⁶]	pELYAFARVa	89 (62–129)	100
Manse-CAP2b[Ala ⁷]	pELYAFPVa	Inactive	
Manse-CAP2b[Ala ⁸]	pELYAFPRVa	3500 (2250–5500)	45

^a Values are derived from dose–response curves based on data points derived from six replicates.

truncated analogs, and a Manse-CAP2b Ala scan series of analogs on housefly Malpighian tubules is presented in Table 1. The fluid secretion activity of *Manduca* CAP2b (EC₅₀ = 53 nM) lies midway between that of the native Musdo-CAP2b/PVK-1 (EC₅₀ = 9 nM) and Musdo-CAP2b/PVK-2 (EC₅₀ = 102 nM); and thus can serve as a reasonable model for a structure-activity relationship study of CAP2b/PVK peptides in the housefly. Interestingly, removal of the pE residue on the N-terminus of Manse-CAP2b leads to the C-terminal heptapeptide sequence common to both Manse-CAP2b and Musdo-CAP2b-1; and the diuretic activity of this fragment Manse-CAP2b[2–8] (EC₅₀ = 8 nM) matches that of Musdo-CAP2b-1 (EC₅₀ = 9 nM). The increase in activity observed with the loss of pGlu¹ likely arises from the fact that it now more closely matches the sequence of the native peptide. Removal of another amino acid residue from the N-terminus, as in Manse-CAP2b[3–8] leads to a large drop of an order of magnitude from the parent *Manduca* CAP2b peptide. Removals of a third (Manse-CAP2b[4–8]) and fourth (Manse-CAP2b[5–8]) residue lead to analogs that retain only trace activity. In the case of the Ala scan series, replacement of the Arg⁷ (Manse-CAP2b[Ala⁷]) led to an analog with no significant activity, indicating that Arg⁷ is therefore the most critical of residues. Replacement of Val⁸ (Manse-CAP2b[Ala⁸]; EC₅₀ = 3500 nM) led to a large drop in activity (70-fold) in comparison with the parent peptide, and demonstrated retention of only 45% of the maximal response of Manse-CAP2b and the native housefly peptides. Residues pGlu¹ (Manse-CAP2b[Ala¹]; EC₅₀ = 23 nM), Tyr³ (Manse-CAP2b[Ala³]; EC₅₀ = 33 nM), Phe⁵ (Manse-CAP2b[Ala⁵]; EC₅₀ = 115 nM), and Pro⁶ (Manse-CAP2b[Ala⁶]; EC₅₀ = 89 nM) would appear not to be critical for housefly diuretic activity as the resulting analogs were either a little more or a little less active. While Manse-CAP2b[Ala⁵] demonstrated a maximal response of 70%, this value is not statistically different from that of the parent peptide. Leu² would appear to be a semicritical residue, as Manse-CAP2b[Ala²] (EC₅₀ = 373 nM), proved to be about six-fold less active.

4. Discussion

The evaluation of the CAP2b sequence from *M. sexta*, Manse-CAP2b, in heterologous Malpighian tubule fluid secretion assays in fruit flies and houseflies [3,5,18] established that this family of peptides demonstrated diuretic activity in addition to cardioacceleratory activity. Seven of the eight residues of Manse-CAP2b are identical with the most potent of the native housefly CAP2b/PVK peptides, Musdo-CAP2b/PVK-1. As can be seen in Table 1, the EC₅₀ for housefly Malpighian tubule fluid secretion of Manse-CAP2b is midway between that of the two CAP2b/PVK peptides native to the housefly; and thus can serve as a relevant model sequence to determine a structure-activity relationship profile. The C-terminal fragment Manse-CAP2b[3–8] retains a significant portion of the Malpighian tubule fluid secretion activity of the parent peptide (Table 1). The active core, the minimum sequence required to retain significant diuretic activity in the housefly, is therefore the C-terminal hexapeptide. However, full activity requires the C-terminal heptapeptide fragment (Manse-CAP2b[2–8]), which is more active than the parent Manse-CAP2b and equipotent with the native Musdo-CAP2b-1 (Table 1). The results of the Ala-replacement series identify two residues, Arg⁷ and Val⁸, as critical for diuretic activity. The residue Leu² is semicritical; whereas all others are not critical. Not surprisingly, the critical (R⁷ and V⁸) and semicritical (L²) residues are among the most conserved among the CAP2b/PVK family throughout arthropods. The most conserved residues among the CAP2b/PVKs are the C-terminal PRVa-mide as well as a Leu at position 7 (equivalent to Leu² in Manse-CAP2b) from the C-terminus [27]. The structure-activity relationship profile determined in this study can aid in the design and development of peptidomimetic agonist/antagonist analogs of this diuretic peptide family with enhanced biostability and bioavailability as tools for arthropod endocrinologists and as potential pest management agents capable of disrupting the water balance in target insects.

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